Child Maltreatment Influences Methylation Density of Serotonin Transporter Gene (5HTTLPR) Depending on Genotype

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Abstract

Methylation of the serotonin transporter gene (5HTTLPR) might be one of the underlying molecular mechanisms of epigenetics through which effects of child maltreatment persist into adulthood. We investigated how the experience of child maltreatment is associated with methylation density of 5HTTLPR and whether 5HTT genotype moderates this relation. The sample consisted of 22 females selected from the larger TwinPAD study (Out, Pieper, Bakermans-Kranenburg & Van IJzendoorn, 2010). DNA was obtained from buccal cells. The percentage of methylation in the first 245 bps was assessed using quantitative mass spectroscopy. Experiences of child maltreatment were established using the Adult Attachment Interview, coded with the Modified Maltreatment Classification System. We found that maltreatment severity was not significantly related to higher methylation density of 5HTTLPR, but the interaction of genotype with maltreatment severity significantly predicted methylation density. For carriers of the *ss* and *sl* genotype, more severe maltreatment was associated with higher methylation density, while for carriers of the *ll* genotype more severe maltreatment was associated with lower methylation density. We conclude that the relation between maltreatment severity and methylation density of 5HTTLPR is moderated by genotype, with *ll* carriers being protected against the methylating effects associated with maltreatment experiences. This study suggests that DNA methylation may be one of the molecular mechanism by which child maltreatment affects current and long-term functioning.

Keywords: DNA methylation, child maltreatment, serotonin transporter gene (5HTTLPR), genotype.

The prevalence of child maltreatment is alarming. In the United Stated 39.5 per 1000 children experienced some form of abuse (Sedlak et al., 2010). For the Netherlands, the prevalence rate was estimated to be 34 per 1000 children (Alink et al., 2010). This is of great concern because experiences of maltreatment in childhood are often associated with a variety of physical, psychological and behavioral problems (Alink, Cicchetti, Kim & Rogosch, 2012; Spann et al., 2012; Teisl & Cicchetti, 2008). In some cases the negative consequences associated with maltreatment continue to affect these individuals into adulthood. Adults who have experienced abuse or neglect more often suffer from health problems and are at increased risk for the development of psychopathology; such as depression and borderline personality disorder (Keyes et al., 2012; Wegman & Stetler, 2009; Widom, Czaja & Paris, 2009; Widom, DuMont & Czaja, 2007). The development of this vulnerability to psychopathology can be explained by a complex interplay between genetic factors and environmental experiences (McCrory, De Brito & Viding, 2010).

Recently, researchers discovered a mechanism that influences this gene environment interaction which is referred to as ‘epigenetics’. The term epigenetics was introduced in the twentieth century. Conrad Waddington (1942) used the term epigenetics for the causal factors by which the genotype transforms into the phenotype, while Nanney (1958) defined epigenetics as changes in genetic material in response to environmental or other stimuli (Haig, 2007). However, at that time the underlying mechanisms were unknown (Franklin & Mansuy, 2010).

Now, epigenetics refers to biochemical modifications that affect gene expression, without altering the primary DNA sequence (Feil & Fraga, 2012). The primary DNA sequence is the structural part of the genome and is relatively stable across the individual’s lifetime (Zhang & Meaney, 2010). In contrast, epigenetic adaptations can occur at any time point during an

organism's life course. Epigenetic processes are present from fertilization and regulate the differentiation of tissues and cells, by silencing some genes while expressing others (Gudsnuk & Champagne, 2011). This process leads to a diversity of cell types in the body; for example, to the transformation of differentiated neurons and blood cells, which are identical in genetic information but significantly different in epigenetic profiles. Epigenetic change is dynamic and allows a flexible response to environmental challenges (Szyf, 2009). Because of this responsiveness to the environment, the epigenetic mechanism offers a way through which the environment can shape the genome and have a lifelong impact on health and behavior (Van IJzendoorn, Bakermans-Kranenburg & Ebstein, 2011). Thus, effects of child maltreatment may persist into adulthood through this epigenetic mechanism.

A gene that is of particular interest in the context of child abuse is the serotonin transporter gene (5HTTLPR, hereafter 5HTT). The 5HTT gene has a critical role in the regulation of emotional states and is one of the best-studied genomic variations in biological psychiatry (Heim & Binder, 2012). The 5HTT polymorphism consists of two common alleles in European populations, a short (*s*) variant with 12 copies of a 22 bp repeat element and a long (*l*) variant which has 14 copies of the repeat element (Philibert et al., 2008). The 5HTTLPR genotype consists of three variants: *ss*, *sl* and *ll*.

The short variant of the 5HTT is associated with less efficient serotonin transport. Carriers of the short allele have been suggested to be more vulnerable to a range of disorders including migraine headaches, major depression, autism, and alcoholism (Conroy et al., 2004; Feinn, Nellissery & Kranzler, 2005; Kiyohara & Yoshimasu, 2010). Furthermore, in a number of gene-by-environment interaction (G x E) studies the 5HTT genotype has been shown to moderate the effects of environmental influences. Most studies support the diathesis-stress model in which

carriers of short alleles are considered to be more vulnerable to negative environmental influences (Zammit & Owen, 2006). For example, Caspi and colleagues (2003) demonstrated that individuals homozygous for short alleles showed more depressive symptoms and suicidality when faced with stressful life events than individuals homozygous for the long alleles did. In addition, short variants have been associated with increased vulnerability to alcohol dependence, posttraumatic stress symptoms, depression and anti-social behavior in the presence of stressors or adversity (Cicchetti, Rogosch & Thibodeau, 2012; Karg, Shedden, Burmeister & Sen, 2011; Kilpatrick et al., 2007; Uher et al., 2011).

However, some meta-analyses have not supported the interaction effect of the short variant of the serotonin transporter and environmental adversity (Munafò, Durrant, Lewis & Flint, 2009; Risch et al., 2009). In fact, there is an increasing inconsistency in the growing G x E literature. Discrepancies in findings may be due to heterogeneity in methodology. One specific determinant that has emerged is the method used to assess environmental adversity (Uher & McGuffin, 2010). Moreover, G x E studies often lack statistical power due to small sample sizes, and the complexity of the analyses increases the risk of false positive findings (Munafò et al., 2009). In addition, critics have expressed their concerns about the replicablity of G x E studies and the tendency to publish significant and novel findings (Duncan & Keller, 2011; Munafò & Flint, 2009).

Although carriers of the *s* allele are more vulnerable to adversity, research also has found that carriers of the *s* allele benefit more from positive environments, including lack of adversity, as compared to individuals homozygous for the long alleles (Belsky et al., 2009; Taylor et al., 2006). This is in line with the model of differential genetic susceptibility, which is defined as the varying susceptibility of individuals with specific genotypes to both negative and positive

environments (Belsky, Bakermans-Kranenburg & Van IJzendoorn, 2007). Van IJzendoorn, Belsky and Bakermans-Kranenburg (2012) recently provided meta-analytic evidence supporting the role of 5HTT in differential susceptibility. Carriers of the *s* allele (*ss/sl*) were more vulnerable to adversity, consistent with the diathesis-stress model, but they also benefit more from a supportive environment as compared to *ll* carriers, consistent with differential susceptibility. Ethnicity was an important moderator; since evidence for differential susceptibility only appeared in studies with Caucasian participants (Van IJzendoorn et al., 2012).

In short, G x E studies using the diathesis-stress model have revealed inconsistent results, and therefore the interaction is not fully understood. There is emerging evidence that methylation might also influence the G x E interaction through gene expression. DNA methylation is one of the most common forms of epigenetic modification and is associated with the silencing of gene transcription (Jeltsch, 2002). A methyl molecule (CH3) is coupled to cytosine, at a CpG site. CpG sites are regions of DNA where a cytosine nucleotide arises next to a guanine nucleotide. The effect of this process on gene transcription appears to occur in two ways (Meaney, 2010). Firstly, methylation of DNA itself may physically impede the binding of transcriptional activators to the DNA sites, thus silencing gene expression. Secondly, methylated CpG islands attract a class of proteins known as methylated-DNA binding proteins. These proteins, in turn, attract a cluster of capping proteins that hinder access to the gene for transcription factors that induce gene expression (Meany, 2010). The methylation pattern on the CpG islands is reproduced each time the gene is copied and thereby the effect of methylation is preserved (Van IJzendoorn et al., 2011).

Some researchers have suggested that the influence of early life experiences on wellbeing in adulthood may be mediated by methylation. More specifically, it is assumed that cellular

signaling pathways activated in response to negative environmental conditions trigger long-term patterns of gene-expression, which in turn influence behavior and health (Heijmans et al., 2008; Heijmans, Tobi, Lumey & Slagboom, 2009; Naumova et al., 2012; Tobi et al., 2009). In this way, early experiences become instantiated in the biological system (Meaney, 2010). Methylation of DNA has recently been studied for several genes, including the serotonin transporter gene. For example, Philibert and colleagues (2007) demonstrated that the degree of methylation was associated with expression levels of the serotonin transporter gene, but only when genotype was taken into account. Furthermore, it has been shown that child abuse was associated with increased methylation of 5HTT promoter region (Beach, Brody, Todorov, Gunter & Philibert, 2010).

Van IJzendoorn and colleagues (2010) demonstrated the possible consequences of methylation in the 5HTT for psychological functioning. Higher levels of methylation were associated with an increased risk of unresolved responses to loss or other trauma in individuals homozygous for the long alleles, while for the carriers of the *ss* variant higher levels of methylation were associated with less unresolved loss or trauma (Van IJzendoorn, Caspers, Bakermans-Kranenburg, Beach & Philibert, 2010).

However, these are only a handful of studies and many details about the process of methylation remain unknown. It seems valuable to add methylation to the gene-by-environment interaction in order to shed more light on the relation between child maltreatment and the 5HTT genotype. If child abuse indeed affects the intracellular milieu, including changes of DNA methylation, it would allow for more insight in how environmental risk mechanisms contribute to developmental outcomes.

In the present study, we expected that the experience of child maltreatment would be associated with methylation density of 5HTT. Firstly, we hypothesized that more severe maltreatment would be related to higher methylation density of 5HTT. Secondly, we hypothesized that this relation of maltreatment severity with methylation density would be moderated by 5HTT genotype. Because studies are inconsistent about the direction of the moderation we did not specify which alleles would be most open to methylation changes. We controlled for age because methylation is known to increase with age (Fraga et al., 2005).

Methods and materials

Participants

Monozygotic female twin pairs were selected from the TwinPAD study. This study investigates caregiving responses and physiological reactivity to infant crying (Out, Pieper, Bakermans-Kranenburg & Van IJzendoorn, 2010). The TwinPAD sample consisted of 50 male and 134 female adult twin pairs. Zygosity of the twin pairs was determined based on a zygosity questionnaire (Magnus, Berg & Nance, 1983), and verified by genetic analyses of six selected polymorphisms. A total of 12 twin pairs (6.5% of the sample) were classified as monozygotic based on the questionnaire while they were in fact dizygotic (Out et al., 2010).

In the present study we used data from monozygotic twin pairs for which complete data on the experience of child maltreatment was available. The sample consisted of 11 monozygotic female twin pairs (n = 22). The majority of the twins were born in the Netherlands (91%) and their mean age was 29.91 (SD = 5.73, range 24 – 43). The mean educational level of the participants was 3.77 (SD = 0.87) on a scale ranging from 1 (elementary school) to 5 (Bachelor's or Master's degree). None of the participants suffered from a mental or physical illness.

Permission for this study was obtained from the Ethics Committee of the Leiden University, and all participants gave informed consent.

Procedure

In 2007 and 2008 twin pairs were invited to Leiden University for a session lasting about three hours. The visit included several cognitive tests, a cry perception task and the Adult Attachment Interview (AAI; Main, Kaplan & Cassidy, 1985). During this visit participants provided buccal cells for genetic analyses, and to verify their zygosity as determined with the zygosity questionnaire. For the present study, which started in 2011, additional DNA was necessary for methylation analyses. Monozygotic female twin pairs were re-contacted by letter or phone and were asked to provide buccal cells for genetic analysis. Swabs were sent by post together with an extensive instruction about how the buccal cells should be collected. Participants returned the swabs by post.

A total of 65 monozygotic twin pairs were approached (n = 130 females). Twenty-two females could not be reached due to moving and the new address was untraceable (n = 16) or residence outside the Netherlands (n = 5), and one person deceased. Another 16 persons were not willing to participate because of disinterest or time constraints. Moreover, 22 females were excluded because they did not return their swabs or their twin sister did not return the swabs. The remaining 70 females (35 twin pairs) returned their swabs. For a subset of 11 pairs (n = 22) methylation analyses were done.

Child maltreatment

Experiences of child maltreatment were derived from the Adult Attachment Interview (AAI; Main et al., 1985). The Adult Attachment Interview is a semi-structured interview focusing on

early attachment experiences and their effects upon current functioning. In this context several questions concerning experiences of abuse are asked. Experiences of child abuse were coded with the Modified Maltreatment Classification System (MMCS; English & the LONGSCAN Investigators, 1997), a modified version of the Maltreatment Classification System (Barnett, Manley & Cichetti, 1993). The MMCS was originally developed as a method of collecting information from Child Protective Services (CPS) records using systematic and reliable criteria (English, Bagdiwala & Runyan, 2005).

The MMCS presents a method for classifying maltreatment including dimensions of subtype, severity, frequency, chronicity, and perpetration of abuse experiences. The MMCS includes five major categories of maltreatment: physical abuse, physical neglect, sexual abuse, emotional maltreatment, and moral-legal/educational maltreatment. In addition, the MMCS includes specification of subtypes of abuse and neglect within these broader categories. For example, under physical neglect the abuse subtypes of failure to provide and lack of supervision are coded. Each subtype of abuse is coded for severity ranging from 1 (*low*) through 5 (*high*) (English et al., 2005). A sixth category of abuse within the coding system concerns caregiver substance use (Litrownik et al., 2005). This category refers to the use of drugs and/or alcohol that has a negative effect on the well-being, caretaking, or safety of the child. Five independent coders were trained to achieve agreement with an expert coder (Dr. Dorothée Out). Subsequently, 10 interviews were selected for a reliability assessment. The interrater reliability, measured by intraclass correlations was almost perfect for the subscales physical abuse (ICC = .82), sexual abuse (ICC = .96) and emotional maltreatment combined with caregiver substance use (ICC = .82). For the subscale physical neglect agreement was moderate (ICC = .54).

In this study two dimensions of the MMCS were used: the occurrence of maltreatment and the severity of the abuse experiences. For the occurrence of maltreatment, participants were divided into two groups; those who had experienced maltreatment in their childhood (up to 18 years) and those who did not have any experience of maltreatment in their childhood. The second approach defined overall maltreatment severity (as described by Litrownik et al., 2005). A single severity score was calculated for each participant reflecting the highest severity level coded across maltreatment types. Substance use of the caregiver was rated as severity level 1. In addition, when a participant had experienced more than one form of maltreatment one point was added. For those participants who had no experiences of abuse a severity score of 0 was applied. Each participant was assigned a severity score between 0 and 6. Ten participants were selected and their severity score was coded by two independent coders, which yielded perfect agreement ($\kappa = 1$).

Genotyping

Genotype of the 5HTTLPR locus was established using a polymerase chain reaction assay (PCR) as previously described (Pieper, Out, Bakermans-Kranenburg & Van IJzendoorn, 2011). Alleles were scored visually uncut: s at 469 bp., l at 512 bp.; cut: s_g at 402 (+67 bp.), l_g at 402 (+110 bp.). The s_g and l_g variants were found to show low transcriptional efficiency; thus, they are similar to s alleles in function (Wendland, Martin, Kruse, Lesch & Murphy, 2006). Therefore, both s_g and l_g variants were classified as s alleles. However, we also performed analyses with the original alleles to control for their influence. For the statistical analyses, genotype was coded as the number of s alleles.

Methylation analysis

DNA was extracted from buccal cells using standard methods. The unmethylated cytosine residues were converted to thymidine using bisulfite modification. The region flanking the previously identified CpG island was then PCR amplified in two contigs using two specific primer sets. Methylation ratios for each of the CpG residues were then determined using quantitative mass spectroscopy by Sequenom (San Diego, California). We used the percentage of methylation in the first 245 bps. The *z*-standardized value was used to facilitate interpretation.

Statistical analysis

To analyze associations between child maltreatment, methylation density, age and 5HTT genotype we used IBM SPSS version 19.0. Data were inspected for outliers and missing values and assumptions underlying the analyses used were evaluated. Bivariate associations were examined for all key variables using Pearson's *r* as well as Spearman's rho (one-tailed), and relations were visually inspected with scatterplots and boxplots. Besides, we conducted independent sample *t*-tests to investigate whether there were significant differences on any of the key variables between participants in the maltreated group versus the non-maltreated group. Cohen's *d* was used as a measure of effect size.

To test whether methylation density of 5HTT significantly differed between participants in the maltreated group versus participants in the non-maltreated group while taken into account the influence of genotype and age, analysis of covariance was conducted. Maltreatment experience was entered as factor, and age and genotype as covariates. For all variables the *z*-standardized values were used to facilitate interpretation. The assumptions underlying analysis of covariance were checked. Partial eta-squared (partial η^2) was used as a measure of effect size.

To test whether maltreatment severity was related to methylation density, while controlling for age and genotype, a multilevel approach was chosen. A multilevel model (SPSS; linear mixed model) was preferred over hierarchical regression because it enables us to adjust for the covariance structure in the data (Twisk, 2006). Since data were from monozygotic twin pairs key variables are more likely to be correlated within twin pairs. In order to model this covariance, we created two new variables. A number between 1 and 11 was assigned to each twin pair. Within a twin pair a 1 or a 2 was assigned to each sister. For all variables the *z*-standardized values were used to facilitate interpretation.

We specified a model without predictors (intercept-only model) and methylation density as dependent variable. We assumed that methylation density varied across twin pairs, therefore twin pair was included as subject variable with random intercept. From this model, we estimated the intra-class correlation (ρ) to determine the degree of dependency among individuals. The intra-class correlation is calculated by dividing the residual variance by the total variance. In the first model we investigated main effects by including sister number, maltreatment severity, age and genotype as fixed effect predictors. Thereafter, we added twin pair as subject variable with random intercept. The models with random intercept and without random intercept were compared to investigate whether allowing the intercept to vary significantly improved the model.

To investigate whether genotype moderated the relation between maltreatment severity and methylation density, we added an interaction term to the above described random-intercept model. This interaction term was calculated from the standardized predictors (maltreatment severity*genotype) and included as fixed effect variable in the model. The same model with unstandardized variables was used to obtain the unstandardized regression coefficients. The values of the unstandardized regression coefficients were evaluated to determine the effect sizes.

Restricted Maximum Likelihood (REML) estimation was used because the number of groups was small. We used the value of Akaike information criterion (AIC) and the Likelihood Ratio test to compare the fit of the different models. The Likelihood Ratio test was also performed using the Maximum Likelihood (ML) estimation to verify the statistics. For all analyses the level of significance was set at $\alpha = 0.05$.

Results

Preliminary analyses

A total of 22 participants were included in the analyses. The participants in the sample were compared with the intended sample (n = 108) and with the group that did participate but was not selected for methylation analyses (n = 48). Participants included in the sample were significantly younger (M = 30.09, SD = 5.56) than individuals that did not participate (M = 38.02, SD = 11.33) and participants excluded from methylation analyses (M = 42.79, SD = 11.76), $t(62.69) = 4.92$, $p <$ 01, $d = 0.9$, $t(67.88) = -6.13$, $p <$ 01, $d = 1.4$ respectively. Genotype was not significantly different for participants in the sample as compared to non-participants or excluded participants, $t(128) = 1.15$, $p = .25$, $d = 0.3$, $t(68) = -1.43$, $p = .16$, $d = 0.4$. No significant differences emerged across these three groups with respect to maltreatment experience, $t(26.29) = 1.80$, $p = .08$, $d = 0.5$, $t(31.91) = -1.83$, $p = .08$, $d = 0.5$. However, effect sizes were medium and indicated approximately 67% overlap between distributions (Cohen, 1988). Maltreatment severity was significantly lower for participants in the sample (M = 1.41, SD = 1.47) as compared to non-participants (M = 2.52, SD = 1.75) and excluded participants (M = 2.43, SD = 1.70), $t(124) = 2.77$, $p < .01$, $d = 0.7$, $t(67) = -2.41$, $p = .02$, $d = 0.6$.

Descriptive statistics for the study variables are presented in Table 1. Means and standard deviations are presented for continuous variables and frequencies are presented for categorical variables. There were no individuals homozygous for the short allele. There were two participants with a sl_g variant and one participant with an $l_g l_g$ variant. There were 12 individuals heterozygous for the short allele (including $n = 3$ ll_g) and six individuals were homozygous for the long allele. The genotype distribution, without the genotypes with a s_g or l_g variant, was in Hardy-Weinberg equilibrium**,** χ^2 (1, n = 16) = 3.31, $p > .05$ (Rodriguez, Gaunt & Day, 2009). The majority of participants experienced some form of maltreatment and the maltreatment severity ranged from 0 to 4. Data were normally distributed for all variables, as indicated by standardized skewness and kurtosis.

Table 1
Means, Standard Deviations and Frequencies of Study Variables
(N = 22)

Variable	Frequencies	*M*	*SD*
Methylation density		4.42	2.12
Age		30.09	5.56
Genotype			
ss	3		
sl	13		
ll	6		
Maltreatment experience			
Yes	8		
No	14		
Maltreatment severity		1.45	1.53

Note. *M* = mean. *SD* = standard deviation

Table 2 presents the intercorrelations among study variables. No differences in patterns of significant associations between Pearson *r* and Spearman's rho were observed, therefore only Pearson's *r* is reported. The moderate correlation of maltreatment severity with methylation

density was marginally significant (r = .32, p = .08), suggesting that more severe maltreatment experiences were related to higher methylation density. However, only 10% (R^2 = .10) of the variance in methylation density is accounted for by maltreatment severity. There was a moderate but non-significant correlation between genotype and age in years (r = .31, p = .08), with a small effect size (R^2 = .10). As presented in Table 3, the independent *t*-tests showed that the maltreated and non-maltreated groups did not differ significantly with respect to methylation density, age or genotype (p > .05).

Table 2
Summary of Intercorrelations among Study Variables.
(N = 22)

Variable	*1*	*2*	*3*	*4*	*5*
1. Methylation density	—	.14	.24	.19	.32
2. Age		—	.31	.17	.001
3. Genotype			—	.14	.07
4. Maltreatment experience				—	.73*
5. Maltreatment severity					—

Note. *p < .05, one-tailed.

Table 3
Independent t-tests for the Differences between the Maltreated- and the Non-Maltreated Group
(N = 22)

	Maltreated (n = 14)		Non-maltreated (n = 8)				Cohen's
	M	*SD*	*M*	*SD*	*t*	*p*	*d*
Methylation density	0.14	1.06	- 0.25	0.90	- 0.88	.39	0.4
Age	30.79	5.22	28.88	6.29	- 0.77	.45	0.3
Genotype	0.93	0.73	0.75	0.46	- 0.62	.54	0.3

Note. M = mean. *SD* = standard deviation, *d* = standardized difference

The analysis of covariance revealed no main effects of age, $F(1, 18) = 0.02$, p = .90, partial η^2 = .001; nor genotype, $F(1, 18) = 0.75$, p = .40, partial η^2 = .04. Moreover, methylation density did not significantly differ between the maltreated and non-maltreated group after

controlling for age and genotype, $F(1,18) = 0.48$, $p = .50$, partial $\eta^2 = .03$. These findings indicate that methylation density of 5HTT was not significantly different for participants with an experience of maltreatment versus participants without any experience of maltreatment.

Next, we tested whether the severity of the maltreatment was related to higher methylation density of the 5HTT, while controlling for the influence of age and genotype. For these analyses the variable genotype was recoded in a new variable based on the number of *s* alleles in the genotype; one or two *s* alleles (carriers of the *ss* and *sl* variant) and no *s* alleles (carriers of the *ll* variant). The intra-class correlation obtained from the intercept-only model was .43. This means that 43% of the total variance in methylation density was explained by variance between twin pairs.

The multilevel model including the fixed effect predictors did not significantly improve when a random intercept for twin pair was added, $\chi^2(1) = 1.60$, $p > .05$. The Likelihood Ratio test using ML estimation revealed similar results. The Akaike's information criterion (AIC) was lower for the random-intercept model (AIC = 59.73) as compared to the model without random intercept (AIC = 61.32). A smaller AIC value indicates a better fit of the model, therefore we interpreted the random-intercept model. The multilevel model with random intercept revealed no significant main effects on methylation density. Age did not significantly predict methylation density, $F(1, 7.12) = 0.17$, $p = .69$, neither did genotype, $F(1, 7.13) = 0.004$, $p = .95$. Maltreatment severity did also not significantly predict methylation density, $F(1, 11.43) = 1.15$, $p = .31$.

To investigate whether genotype interacted with maltreatment severity in predicting methylation density, the interaction term was added to the model. Adding the interaction improved the model as indicated by a decrease in Akaike's information criterion (AIC = 54.12).

The Likelihood Ratio test revealed that this improvement was significant, $\chi^2(1) = 5.61$, $p < .05$, which was confirmed by the Likelihood statistic calculated with ML estimation. Parameter estimates are shown in Table 4. Again age did not significantly predict methylation density, $F(1, 7.23 = 1.29$, $p = .29$. In addition, the relative small value of the unstandardized regression coefficient indicated a small effect size. Genotype did not significantly predict methylation density, $F(1, 6.76) = 0.001$, $p = .98$ neither did maltreatment severity, $F(1, 14.97) = 0.28$, $p = 60$. However, the large values of the unstandardized regression coefficients indicated a large effect size.

The interaction of genotype and maltreatment severity significantly predicted methylation density, $F(1, 15.54) = 5.75$, $p = .03$. The value of the unstandardized regression coefficient was relatively large, which indicated a large effect size. The interaction effect indicated that carriers of the *ss/sl* genotype and carriers of the *ll* genotype were differently affected by maltreatment severity. The same analyses corrected for l_g and s_g alleles yielded similar results.

Table 4
Regression coefficients estimated for the Multilevel Model with Interaction

	b	*SE(b)*	*β*	*SE(β)*
Age	0.10	0.09	0.27	.24
Genotype	- 2.68	1.56	0.02	.51
Maltreatment severity	- 1.16	0.73	- 0.85	.53
Genotype*Maltreatment severity	1.91	0.80	1.39	.58

Note. Dependent variable = methylation density, *b* = unstandardized regression coefficient, obtained from the model with unstandardized variables, *β* = standardized regression coefficient, obtained from the model with *z*-standardized variables.

To explore this interaction effect, the sample was split based on the recoded genotype variable. The correlation of maltreatment severity with methylation density was calculated separately for carriers of the *ss* and *sl* genotype and for carriers of the *ll* genotype. The

correlation of maltreatment severity with methylation density was significant for both genotypes. However, for the *ss* and *sl* carriers a positive correlation emerged, $r(16) = .51$, $p = .02$, while for *ll* carriers maltreatment severity was negatively associated with methylation density, $r(6) = -.82$, $p = .02$. These correlations were significantly different ($z = 2.66$, $p = .008$). The interaction graph in Figure 1 shows that for carriers of the *ss* and *sl* genotype a higher maltreatment severity was associated with higher methylation density of 5HTT. For the carriers of the *ll* genotype a higher maltreatment severity was related to lower methylation density of 5HTT.

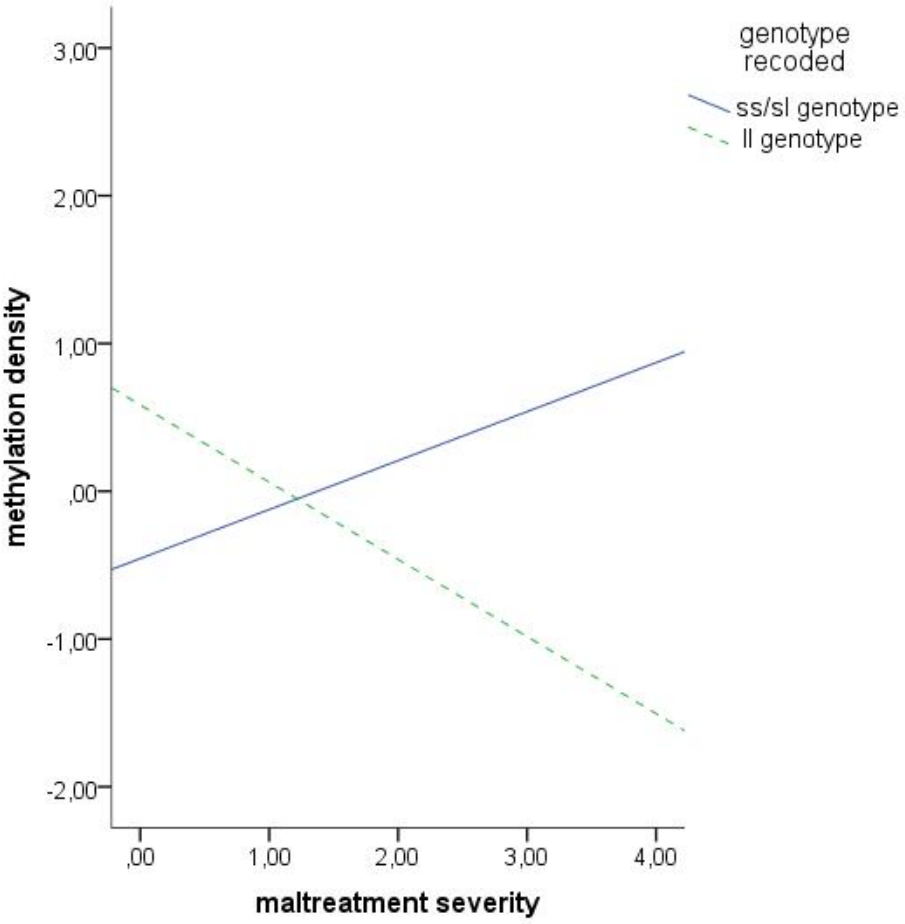

Figure 1. Relation between Maltreatment Severity and Methylation density for *ss/sl* and *ll* carriers.

Discussion

The goal of this study was to investigate the association between child maltreatment and methylation density of 5HTT. We could not confirm the hypothesis that more severe maltreatment relates to higher methylation density of 5HTT. However, the interaction between genotype and maltreatment severity significantly predicted methylation density. For carriers of the *ss* and *sl* genotype, more severe maltreatment was associated with higher methylation density, while for carriers of the *ll* genotype more severe maltreatment was associated with lower methylation density. We found no influence of age on methylation density.

The lack of main effect of maltreatment severity on methylation density of 5HTT contrasts with previous findings. For instance, Beach and colleagues (2010, 2011) showed that reports of abuse during childhood were associated with increased methylation density of 5HTT promoter region. They replicated this for females who experienced childhood sexual abuse. The moderating role of genotype might be responsible for our non-significant main effect of maltreatment severity. The association of maltreatment severity with methylation density was opposite for *ss/sl* and *ll* genotypes. Analyzing both genotypes within one group leads to the non-significant effect. Therefore, it seems important to include genotype when investigating the relation of child maltreatment with methylation density of 5HTT.

To the best of our knowledge, only Vijayendran and colleagues (2012) investigated the effect of child abuse on methylation of 5HTT and included genotype. They reported a significant interaction effect of genotype with childhood sexual abuse on methylation at a specific CpG residue (Vijayendran, Beach, Plume, Brody & Philibert, 2012). Unfortunately, the authors have not interpreted this interaction effect, therefore the role of genotype in the relation of child abuse with methylation density of 5HTT is still unclear.

Research on molecular epigenetics has shown that methylation levels can be different for the *s* allele and the *l* allele. For example, Philibert and colleagues (2007) showed that the degree of methylation was associated with expression levels of the serotonin transporter gene only when controlled for genotype. They showed a trend indicating that the *l* allele was associated with lower methylation density than the *s* allele. Sugawara and colleagues (2011) obtained a similar result; increased methylation was associated with decreased expression levels of the serotonin transporter gene, only for individuals homozygous for the *s* allele.

Although methylation of 5HTT was not significantly influenced by age in the current study, previous research has established that DNA methylation increases with age (Fraga et al., 2005; Bell et al., 2012). Characteristics of our sample and research design might be responsible for this outcome. Our sample was small with a narrow age range (25-43 years), while large samples and wide age ranges are important to detect age-related methylation differences. For example, Talens and colleagues (2012) found age-related increases of DNA methylation in a sample of 460 individuals, aged between 18 and 89 years. In addition, individual differences in DNA methylation are large and age-related changes can occur in a direction that differs per individual. Therefore, it is important to use a longitudinal design, especially when sample sizes are limited (Bjornsson et al., 2008; Wong et al., 2010).

Our sample consisted of monozygotic twins that provided us with the unique opportunity to study methylation within participants with the same DNA. Unfortunately, we have not used the twin design because statistical analyses revealed that within-pair differences with respect to maltreatment severity and methylation density were negligible relative to between-pair differences. The small number of twin pairs might be responsible for this outcome. However, incorporation of the MZ twin design would enhance the opportunities for this study. A

monozygotic twin design is extremely informative in molecular genetic studies (Boomsma, Busjahn & Peltonen, 2002). Firstly, comparisons within- and between twin pairs can be used to specify the extent of epigenetic heritability and stability across the genome. Secondly, we can investigate how differential exposure to environmental factors influence epigenetic mechanisms such as DNA methylation, which in turn may contribute to phenotypic differences in genetically identical individuals (Bell & Spector, 2011; Fraga et al., 2005; Heijmans, Kremer, Tobi, Boomsma & Slagboom, 2007). Thus, it is recommended that the next phase of this study include the other monozygotic twin pairs for which DNA material is already available. Besides, it would be possible to investigate how within-pair differences in experiences of child maltreatment are associated with within-pair differences in methylation density.

In the current study, we investigated associations between child maltreatment, methylation density of 5HTT and genotype. This is an important first step in the investigation of methylation of 5HTT as underlying mechanism that transfers effects of child maltreatment into adulthood. Other researchers have focused on the consequences of 5HTT methylation for psychological functioning in adulthood, rather than early adversity predicting changes in 5HTT methylation (i.e. Park et al., 2011; Philibert et al., 2008). For example, Koenen and colleagues (2011) found that the experience of trauma was related to increased risk of developing post-traumatic stress disorder, but only when 5HTT methylation levels were low.

In a study by Van IJzendoorn and colleagues (2010) higher levels of methylation were associated with a decreased risk of unresolved loss or trauma in carriers of the *ss* genotype, while the relation was opposite for carriers of the *ll* genotype. The authors suggest that the vulnerability of the *ss* variant for the development of psychological problems may be decreased by higher levels of methylation. Compared with our current findings showing that more severe

maltreatment is associated with higher methylation density only in *s* allele carriers, we might argue that methylation may act as a *mechanism* that controls gene-expression in response to early adversity, to protect the individual for maladaptive functioning in adulthood. However, this would not be in line with findings of Beach and collegues (2011) who reported that increased methylation in response to child abuse was associated with more symptoms of antisocial behavior in carriers of the *s* allele.

Obviously, these studies differ with respect to the sample investigated and the method used therefore we cannot draw any definite conclusion. However, we might speculate that methylation might be a mechanism through which different variants of the same genotype become more similar in functioning. Methylation levels vary for different genotypes, which might be to protect individuals for the development of mental problems in response to child maltreatment. Only a handful of studies on this topic have been reported thus far and many methodological issues have to be solved. At the same time, the relation between environmental influences, genetics, and epigenetics is very complex. Therefore, future research is needed to understand the complex interplay between environment and (epi) genetic factors. With respect to DNA methylation, genome-scale studies offer a promising direction, because it includes large samples and provides information about methylation density across the entire genome (Suzuki & Bird, 2008).

Our study has some limitations. Firstly, DNA material to assess methylation was obtained from buccal cells and not from the central nervous system. It is not yet clear to what extent DNA methylation levels are comparable across saliva, blood, and brain tissue (Heijmans & Mill, 2012). Talens and colleagues (2010) compared the methylation levels of candidate loci in blood and buccal cells and found that DNA methylation measured in blood was a strong marker for methylation in buccal cells. However, the question remains whether these DNA methylation

levels are representative for DNA methylation levels found in brain tissue (Heijmans & Mill, 2012). Future studies are needed that compare methylation levels across different tissues. A second limitation concerns the generalizability of our findings. Females in our sample were significantly younger and had experienced less severe maltreatment in childhood, as compared to females who were not in our sample. Furthermore, the majority of participants were Caucasian. Replication in larger samples and in other ethnicities is necessary to confirm the generalizability of our findings.

We conclude that the association between child maltreatment and methylation density of 5HTT depends on genotype. This study suggests that DNA methylation is one of the molecular mechanisms through which early adversity such as child maltreatment, may affect (mal) adaptive functioning in adulthood.

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